kDNA real-time PCR qPCR

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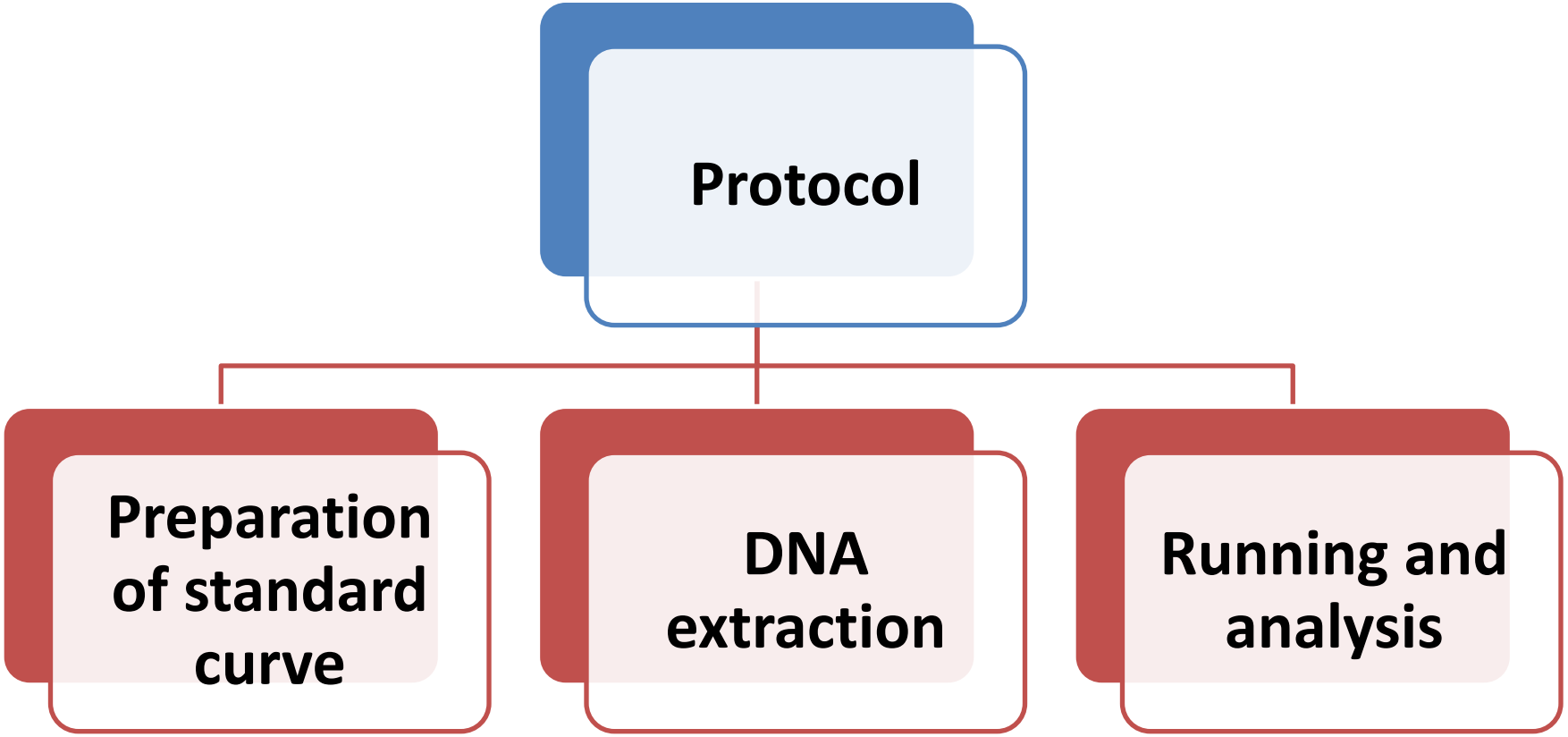
Background:

- **Detection and quantification of the parasite load in samples, using primers (JW11, JW12) chosen in the conserved sequences of the minicircles of kinetoplastic DNA (*Nicolas et al., 2000*).**
- **In Leishmania spp., minicircles are generally present at around 10,000 copies per kinetoplast *(Brewster et al., 1998)*.**

When to use?

- **Estimate the parasite load (number/ml) in blood samples or tissue biopsies of infected individuals.**
- **The assay can detect 1 parasite per ml of blood.**


Protocol
Preparation of standard curve
DNA extraction
Running and analysis

Samples:

- 3 Blood samples.
- 2 Biopsy samples.
- 2 Sand fly females blood fed.

A. Setting up the *Leishmania standard curve:*

1 - Using an inverted microscope examine the *Leishmania tarentolae (non-pathogenic) culture* → Count the number of promastigotes → Calculate the total number of parasites/ml .

2- Prepare different concentrations of promastigotes in 1ml of sterile (PBS), starting from 10^7 and no parasite.

B - DNA extraction of parasites:

- Punch 2 discs from each filter paper samples to be tested as well as standards and place Into separate tubes (about 0.5 mm width).
- Add 180µl PBS and 20µl proteinase K, and then proceed with the DNA extraction using the Qiagene kit protocol.

C. Running the assay:

- **Idea:** *The detection of the PCR amplification products is based on detecting the fluorescent SYBR green dye once bound to the newly amplified double stranded DNA molecules.*

Reaction mixture needed to prepare one “20µl” reaction:

Material	Quantity
2X Absolute blue SYBR Green	10µl
Forward primer (1µM)	1µl
Reverse primer (1µM)	1µl
Extracted DNA	2µl
DDH2O	6µl

Procedure:

- Count the number of the samples to be analyzed including the standard curve and negative controls (*8 points of different DNA concentrations used in creating the standard curve, two negative control, plus the unknown samples to be tested*).
- Prepare M.M.
- Add M.M. to each reaction well (18 μl).
- Add DNA sample (2 μl), including samples from the standard curve DNA.
- Run the qPCR machine.

qPCR thermal cycling program:

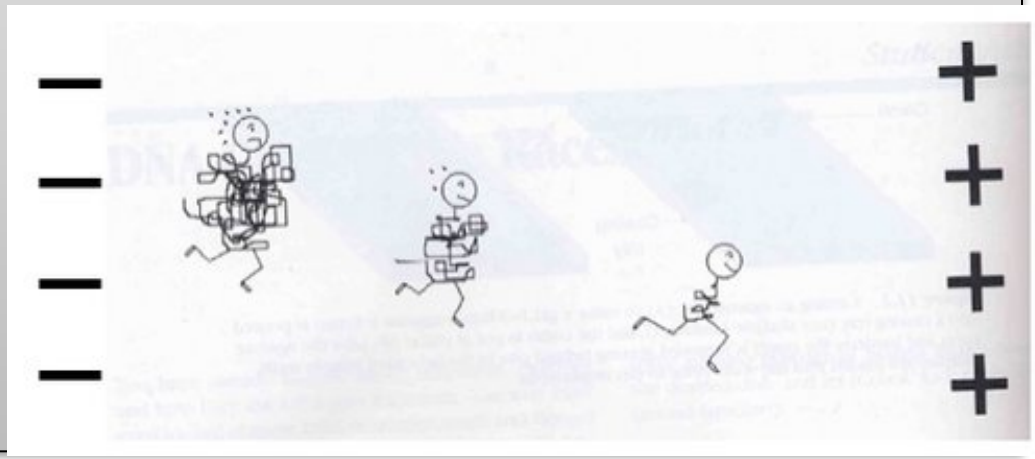
Step	Temp.	Time	Number of cycles
Enzyme activation	95 °C	15 min.	1 cycle
Denaturation	95 °C	10s	40 cycles
Annealing	58 °C	10s	
Extension	72 °C	20s	

Take care:

- ***The forward and reverse primers are diluted in a final concentration of 10 µM.***
- ***Negative control samples receive 2 µl DDH2O.***

Advantages:

- More sensitive (huge amount of KDNA to be amplified).
- No Gel.
- Quantification of parasite load.

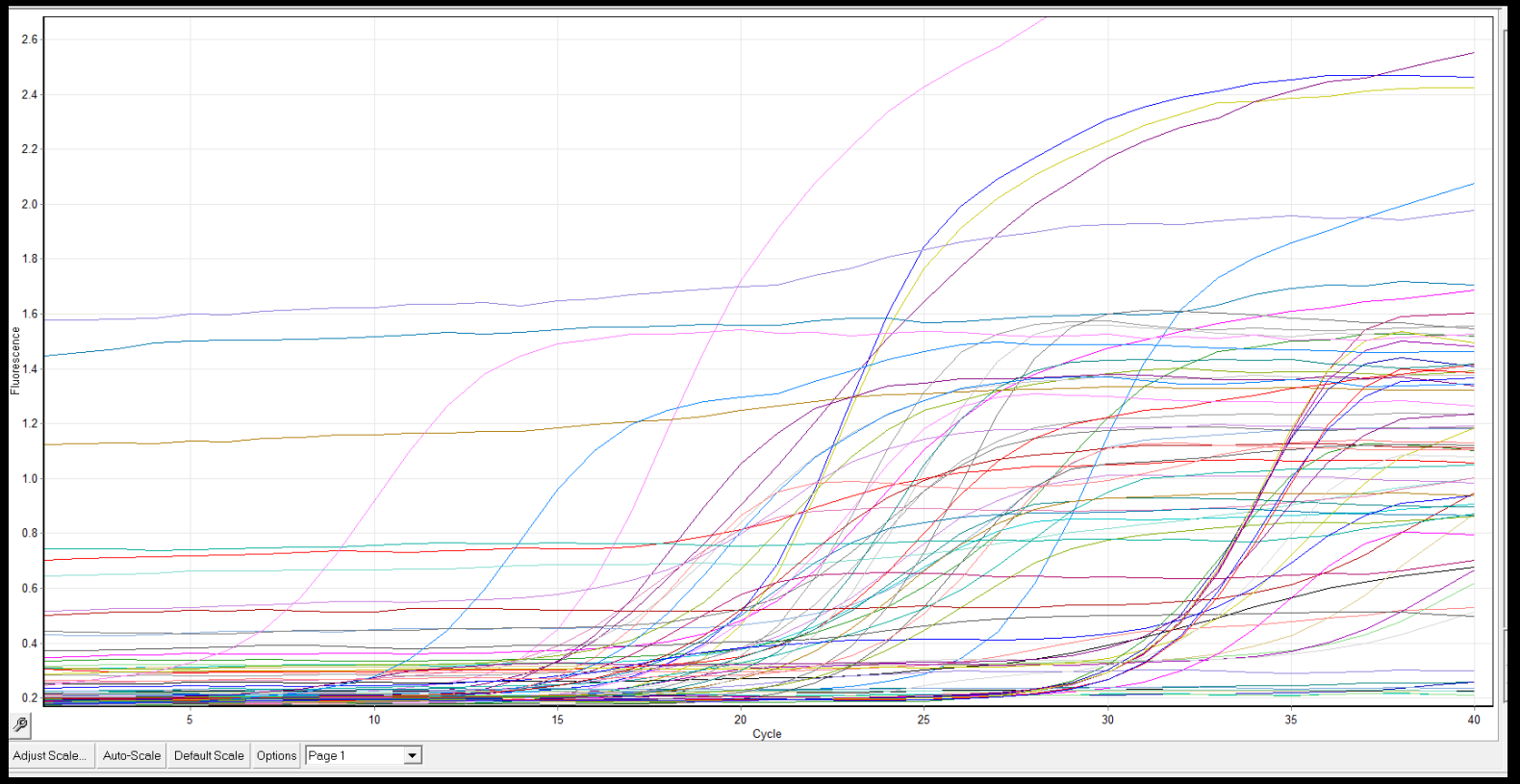


Disadvantages:

- No species identification.
- Primers used can also amplify Trypanosomes (never specific).

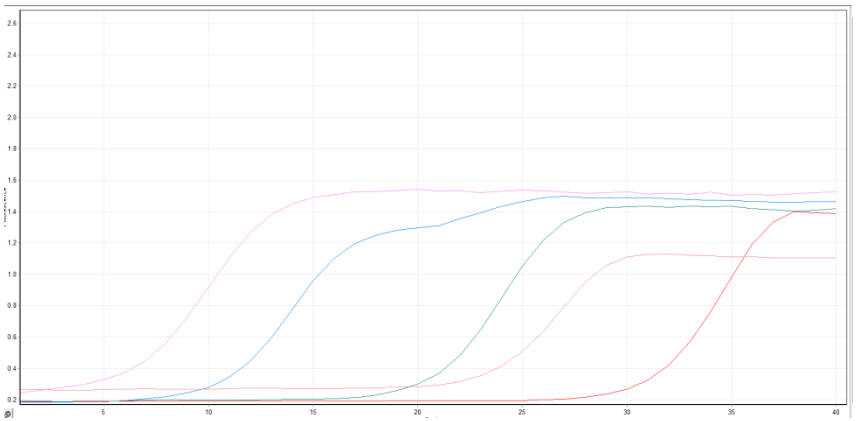
Results

Amplification plot curves:

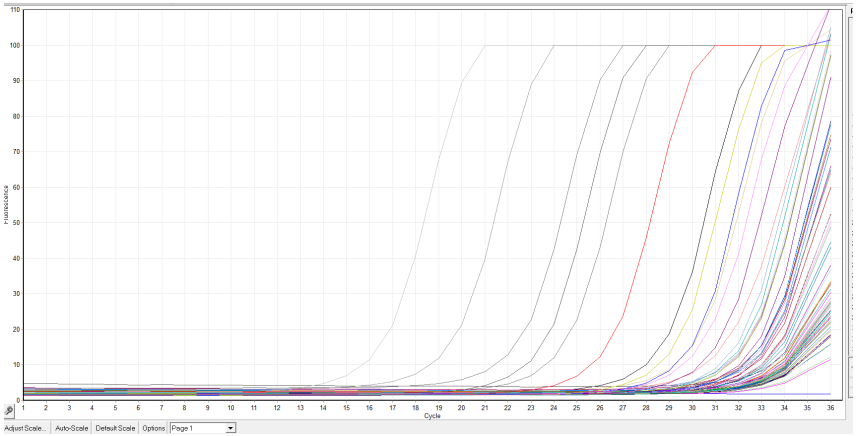


Standard curve for qPCR

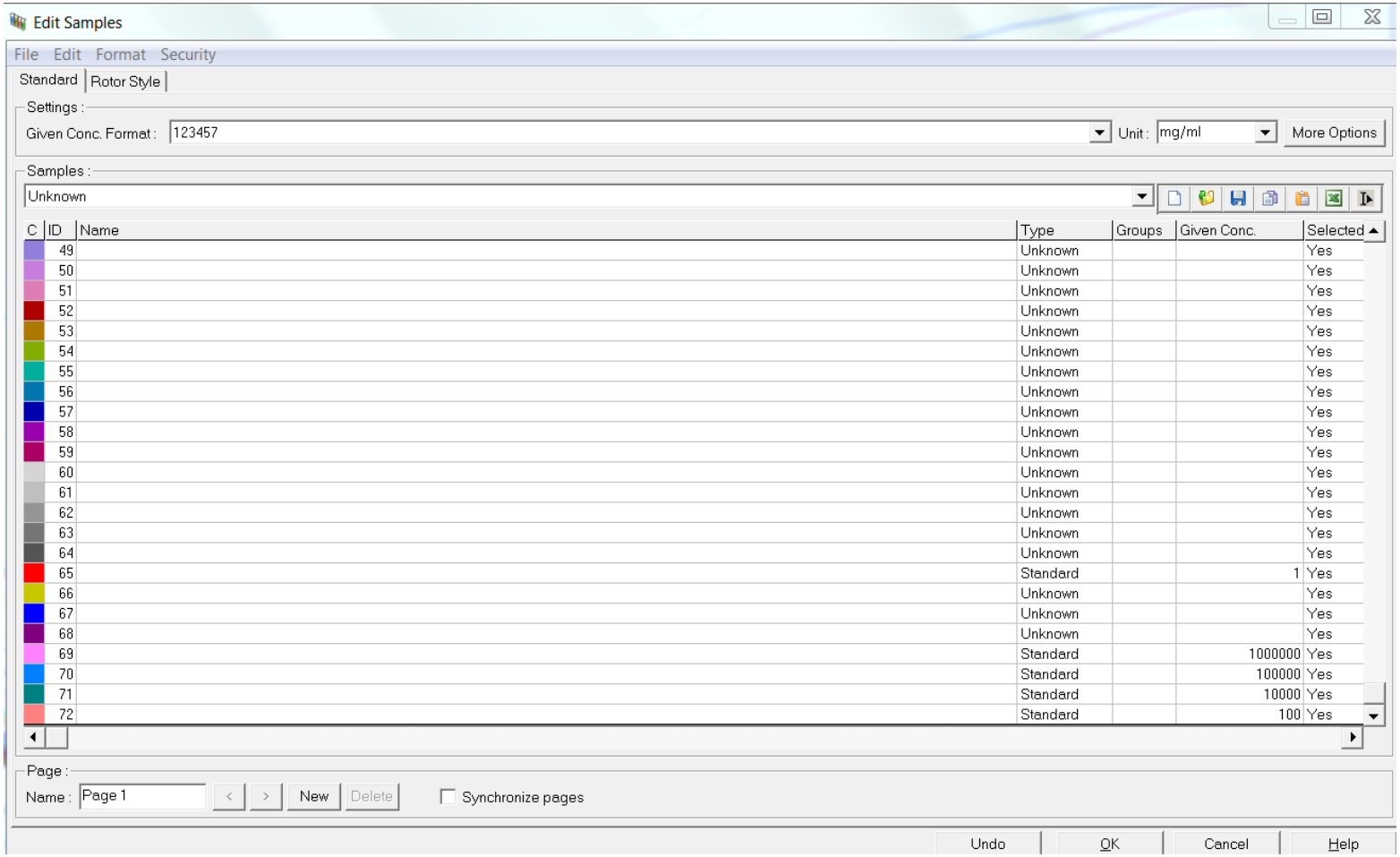
Standard curve for current assay



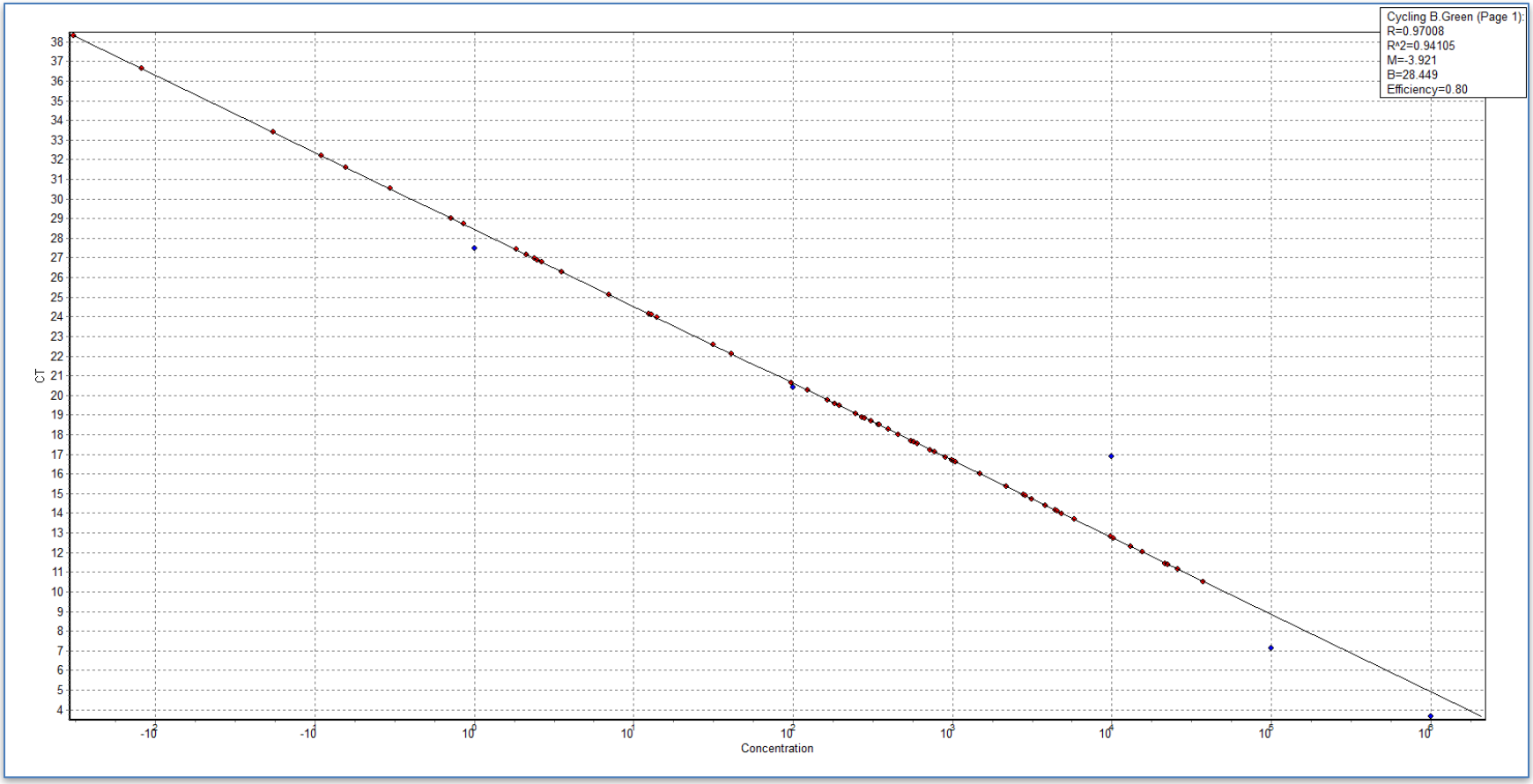
Typical standard curve

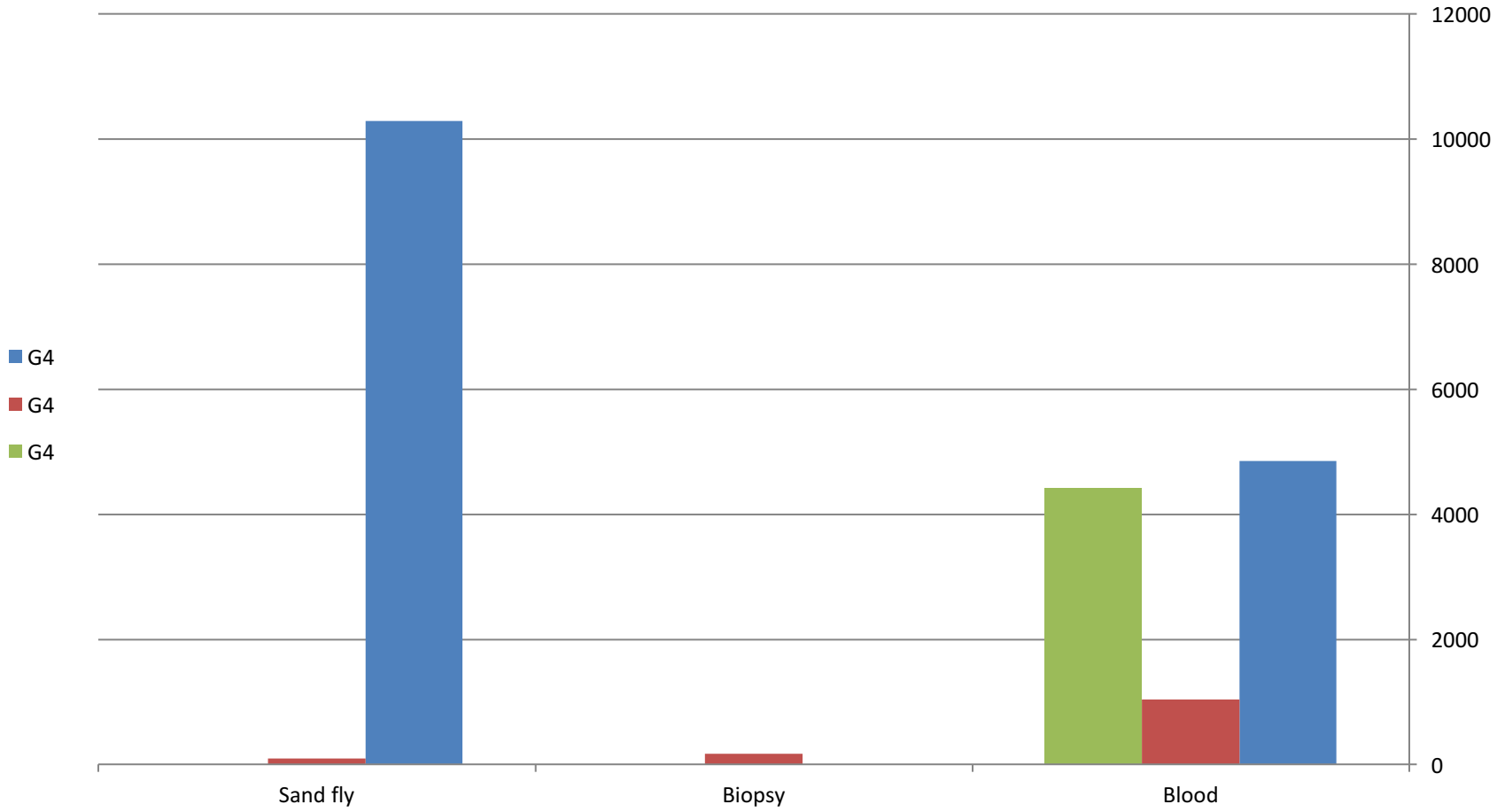


Different concentrations from positive control:



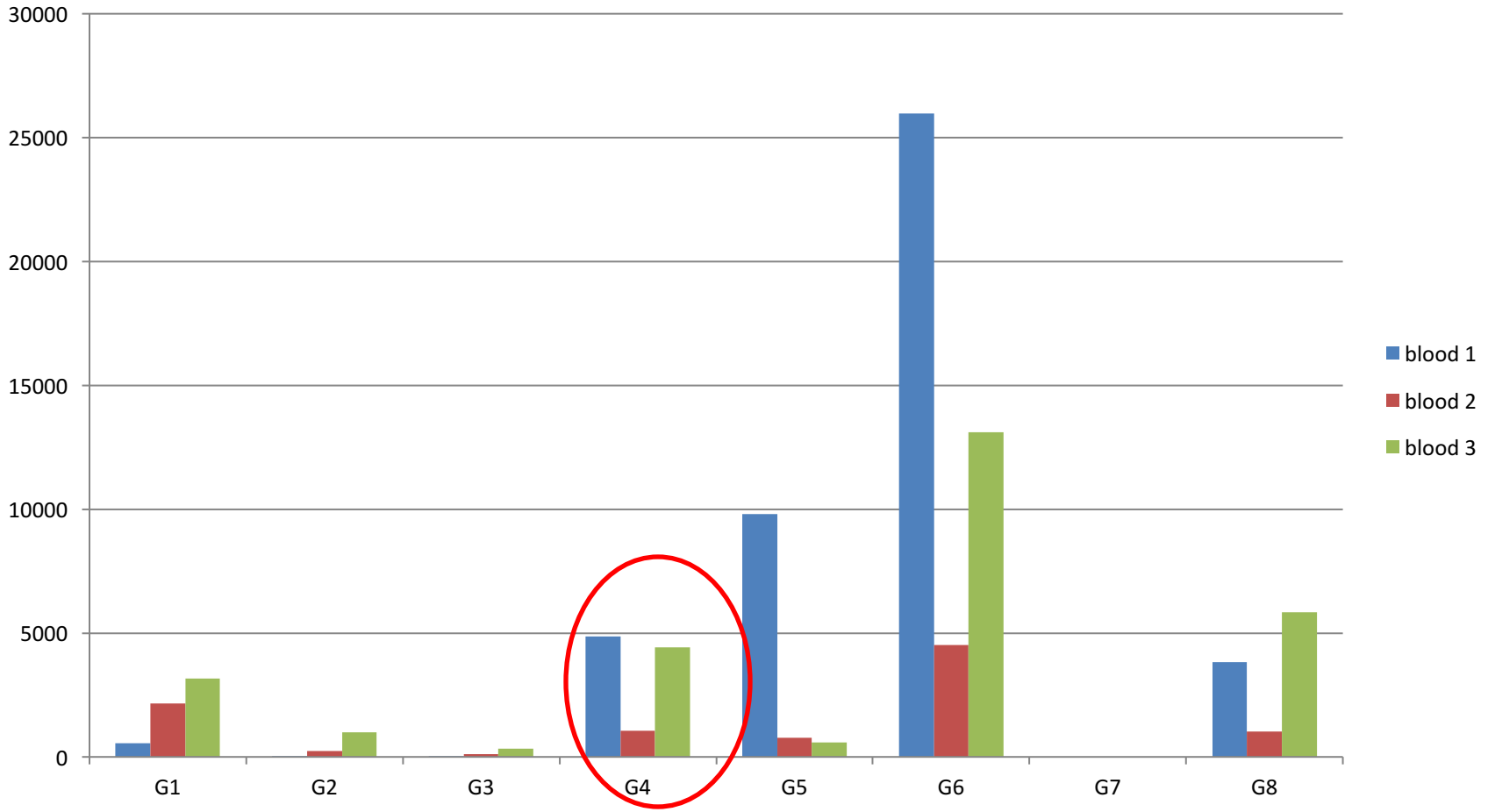
Calibration curve:





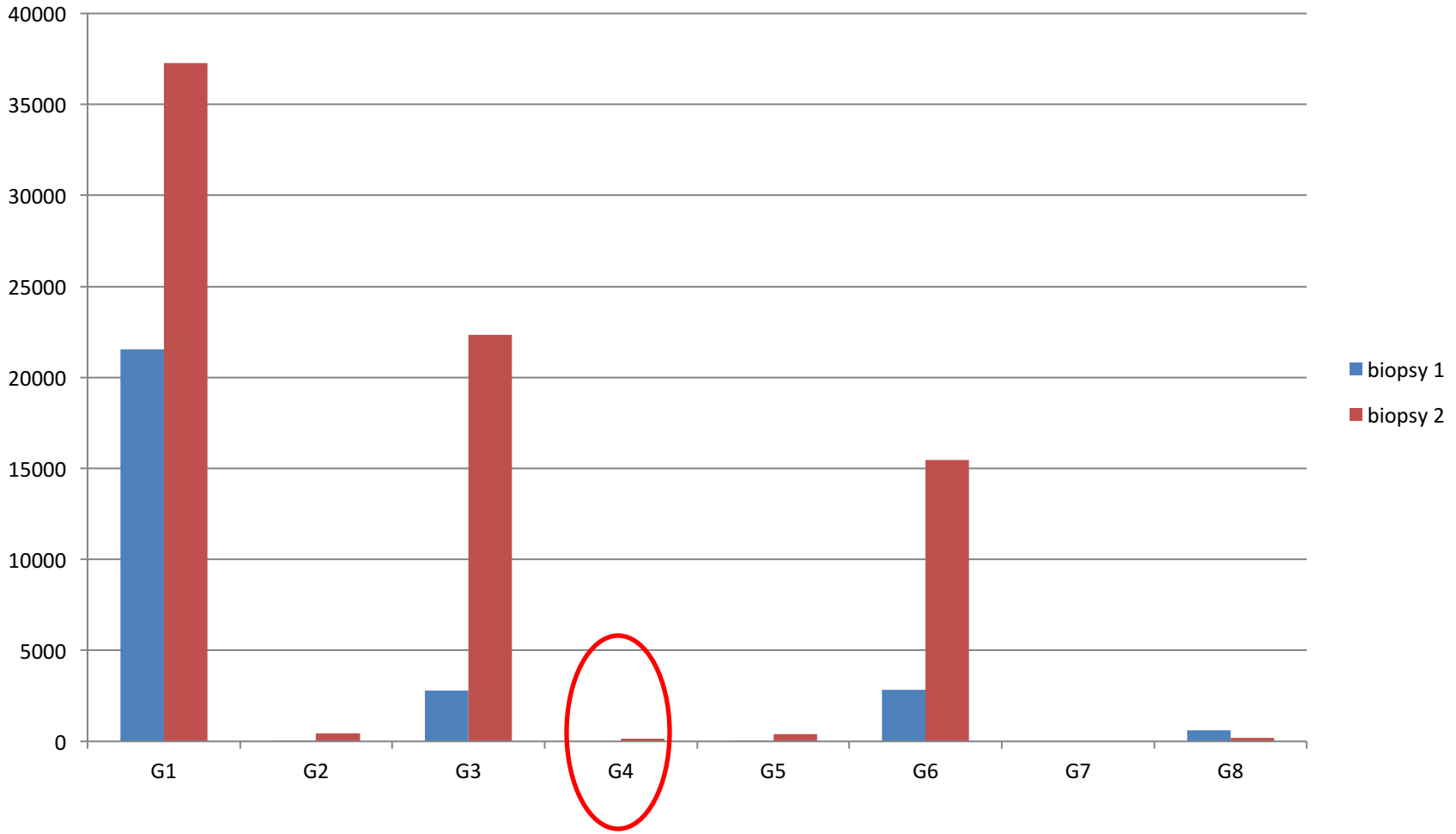
Parasite load detected in different types of samples

Blood samples



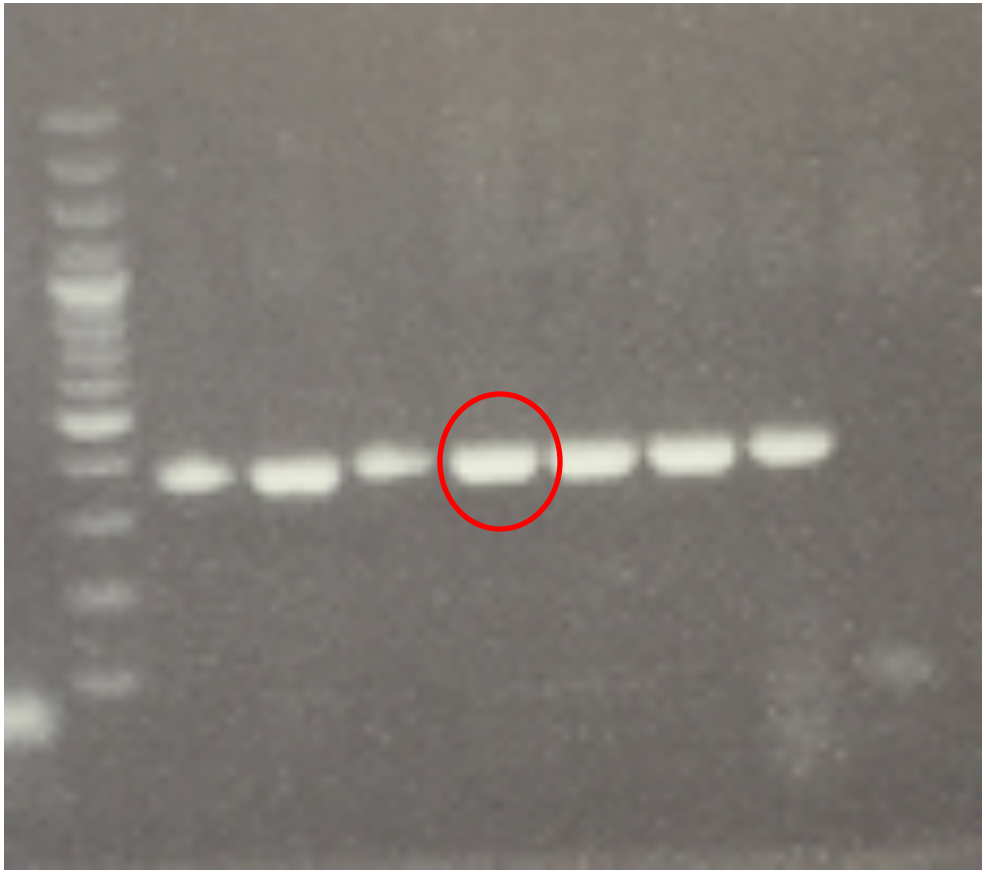
kDNA detected in 100% of blood samples.

Biopsy samples



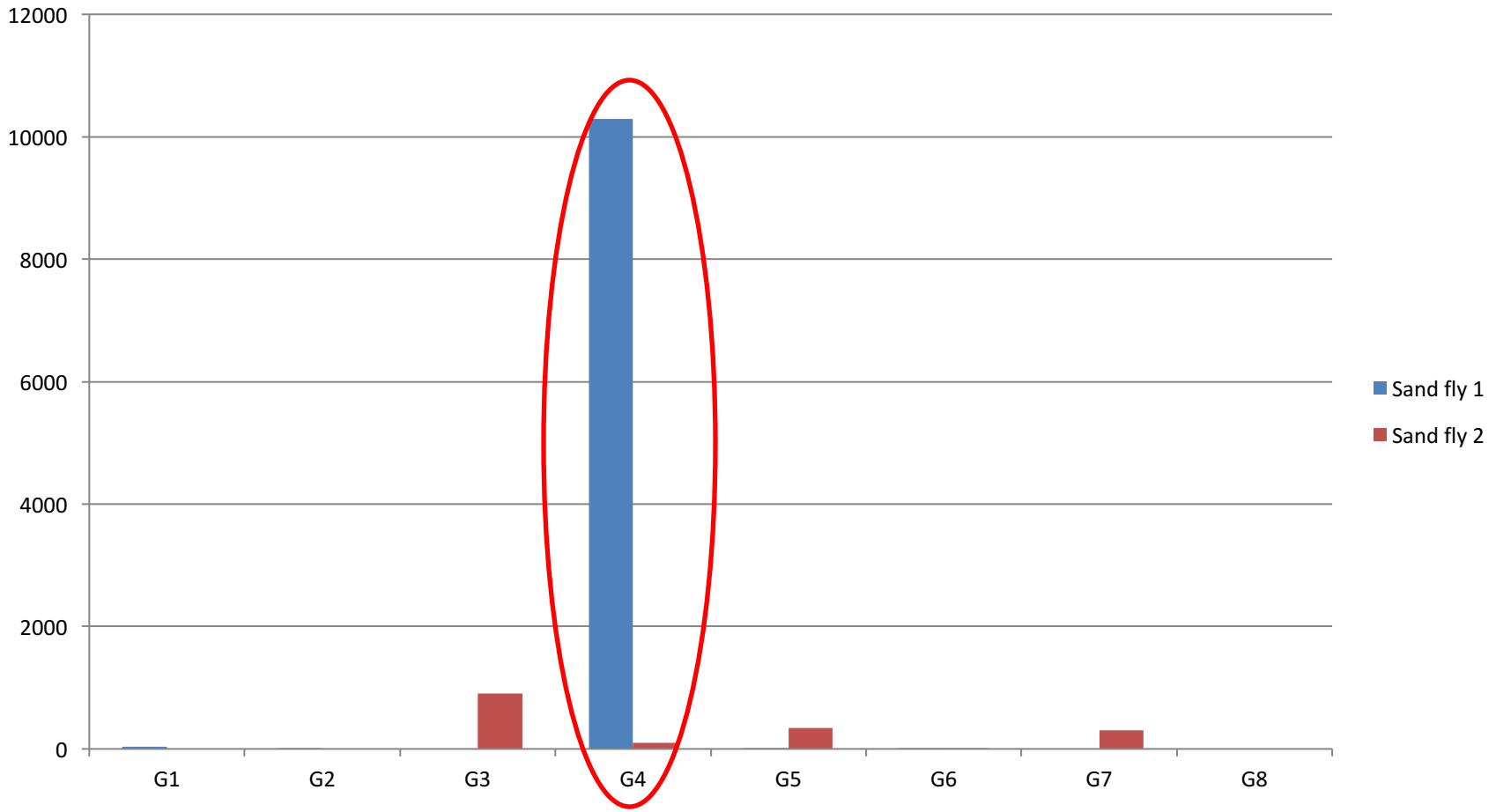
kDNA detected in 50% of Biopsy samples.

Leishmania CytB-PCR



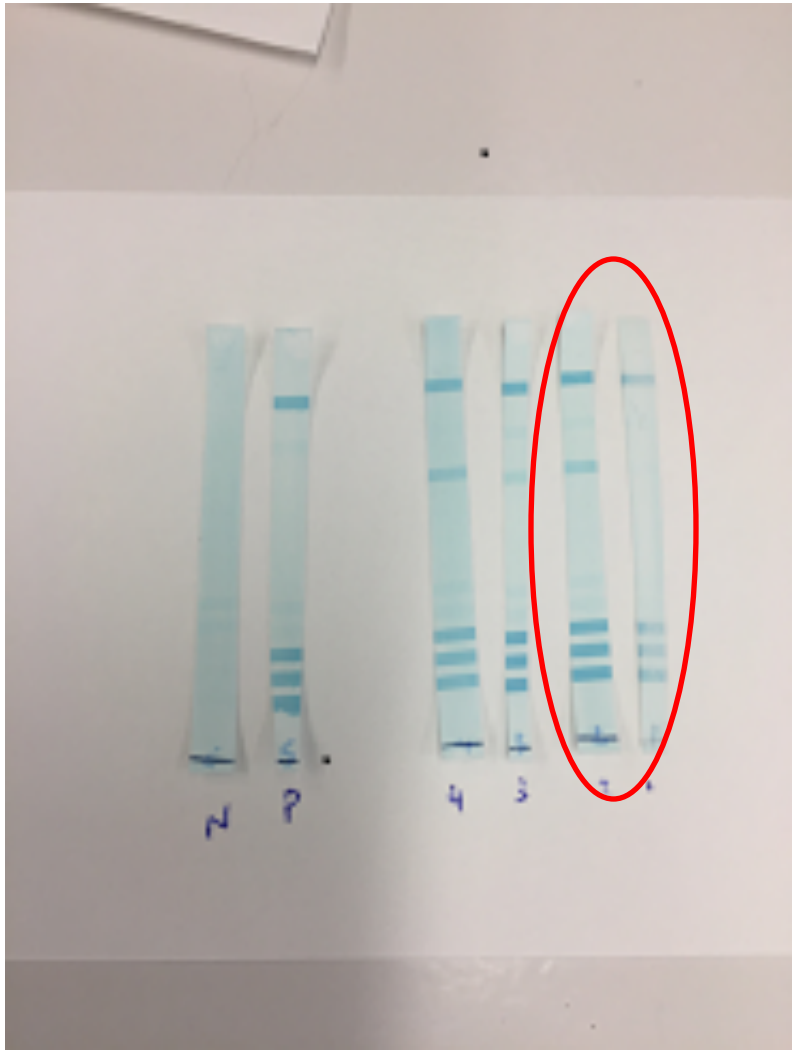
G4	4853	blood
G4	1043	blood
G4	4422	blood
G4	0	Biopsy
G4	164	Biopsy
G4	10293	SF
G4	98	SF

Sand fly samples



kDNA detected in 100% of Sand fly samples.

Reverse line blot



Conclusion:

1- Higher parasite load in Sand fly samples, followed by blood samples with least parasite number detected in Biopsy samples.

2- Anthropophagic nature of tested sand fly samples (detected by RLB).

Thank You